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PROTEIN CHEMISTRY OF NECROTIC WOUNDS.(U)  
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VIRGINIA MASON RESEARCH CENTER  
SEATTLE, WASHINGTON

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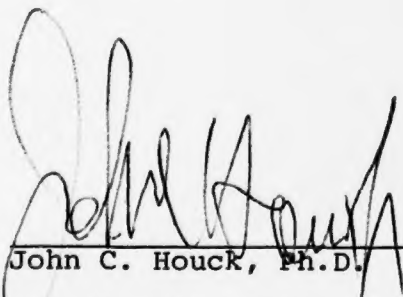
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Final Progress Report for ONR Contract N00014-71-C-0203  
on PROTEIN CHEMISTRY OF NECROTIC WOUNDS

For the Period: October 31, 1975 to October 31, 1976

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During the past year we have isolated and purified and chemically characterized macrophage migration inhibitory factor (MIF), one of the more important lymphokines released from transforming lymphocytes. The fact that lymphocytes release a material which would inhibit the migration of macrophages in culture has been known for perhaps two decades. All attempts to purify this active principle to electrophoretic homogeneity have, until now, been unsuccessful. The primary difficulties involved in the successful purification of MIF activity was firstly the source of the activity, usually the medium obtained from transformed lymphocytes in vitro, and secondly, that MIF is a molecule extraordinarily subject to proteolytic digestion and destruction.

These problems have been resolved by our finding firstly that aqueous extracts of the spleen from immunologically sophisticated animals, including vaccinated calves, contain a sialo-protein which inhibited macrophage migration in vitro and had other properties similar to that demonstrated by classically obtained MIF. Secondly, during the alcohol fractionation of these extracts, we found that essentially all of the proteolytic activity in this lymphoid tissue was precipitable at 50% ethanol, whereas the MIF activity was soluble at this concentration of ethanol and precipitable at 70% ethanol. In this fashion then, large quantities of starting material could be obtained which could be easily separated from contaminating proteases. Because of these advantages, we were then able to use the techniques of membrane ultrafiltration, isoelectric focusing and preparative acrylamide gel electrophoresis to prepare, for the first time, completely homogeneous preparations of MIF activity. This purified material would work both in vivo and in vitro in sub-microgram amounts, was found not to possess protease activity (as has been suggested for cruder preparations) and not to be chemotactic for macrophages, but rather to activate the macrophage phagocytically so that its primary function would be performed at a much-increased rate, and as a consequence of this, the macrophage would not migrate further. In human analogy, if you are already involved in a good meal, why would you want to leave the room and go down the street to the nearest restaurant? Thus, MIF really should be called Macrophage Activating Factor, or MAF.

The molecular weight of this material was determined using both SDS acrylamide gel electrophoresis and exclusion chromatography, and found to be 36,500. Only two carbohydrates were found in the molecule itself; firstly sialic acid and secondly, 0-methylglucopyranoside, a rather rare carbohydrate for most glycoproteins. However, when three-dimensional models of 0-methylglucopyranoside are constructed, it can be seen to resemble certain methyl pentoses particularly, fucose. It has been shown previously, by others, that fucose will inhibit MIF activity in vitro. We suggest that the binding sites on the macrophage for fucose are, in fact, more

specifically addressed to accomplish the binding of 0-methylglucopyranoside. 0-methylglucopyranoside, not unreasonably, is a very effective inhibitor of MIF activity in vitro and in vivo.

We have also discovered, purified and characterized, in part, a new lymphokine, namely "lymphotactin". This is a small glycoprotein which is specifically chemotactic for lymphocytes, as opposed to macrophages, or polymorphonuclear leukocytes. The products of the activation of complement which are known to be specifically chemotactic for polys and, to a lesser extent, macrophages, have no influence whatsoever upon lymphocytes in vitro. However, acting upon a report by Peter Ward that transformed lymphocytes release a material apparently chemotactically stimulating to lymphocytes, we found that our extracts of the thymus from immunologically-sophisticated calves (vaccinated) also contain a material which would in vitro chemotactically attract lymphocytes across the small holes in a Boyden Chamber Millipore filter. This activity had an isoelectric point of 5.9 and was shown to have a molecular weight by both SDS electrophoresis and exclusion chromatography of 10,500 daltons. Its activity is destroyed by incubation with sialadase or proteases, and the material was found to be thermal label.

Purified lymphotactin at sub-microgram amounts would, when injected into the peritoneum of guinea pigs, produce a massive accumulation of lymphocytes IP within as little as four hours! Differential cell counts of these peritoneal cells obtained by lavage, indicated that practically all of these cells were lymphocytes with very few polys or glass-adhering phagocytic mononuclear cells.

Finally, we are currently in the process of characterizing the lymphokine called "Skin Reactive Factor" (SRF) which we have recently purified and which is probably not a lymphokine at all. Our evidence suggests that most lysosomes contain a material with an isoelectric point of 4.2 and a small amount of proteolytic activity against denatured hemaglobin maximally active at pH 3 which, when injected into the skin of rats, causes a marked increase in permeability. This skin permeability factor, analogous to "Skin Reactive Factor", has a molecular weight of between 50,000 and 100,000 daltons and is inhibited by pepstatin, an acid hydrolase inhibitor obtained from fermentation products. These properties of molecular weight, inhibition by pepstatin, isoelectric point and pH optima on denatured hemaglobin are shared by Skin Reactive Factor, Lymph Node Permeability Factor and extracts of every tissue reasonably rich in lysosomes, particularly lymphoid tissues. The belief that SRF is a lymphokine proceeds almost entirely from the fact that normal lymphocytes do not normally have lysosomes. These lysosomes are produced only during the transformation of small lymphocytes to large lymphoblasts and hence, their appearance kinetically coincides with the release of lymphokines.



The mechanism of action of this permeability-producing factor from lymphocyte supernatants or extracts of lymphoid tissue, or extracts of lysosomes, is unknown. It could proceed simply by the degranulation of adjacent mast cells to release histamine or, it could proceed, as we suspect is the case, by virtue of the activation of the acid-leukokinin system described by Lowell Greenbaum. Greenbaum and ourselves have shown that the ground substance of skin is extremely rich in the macromolecular kininogen from which acid leukokinin is released by acid protease activity.

There is very good reason to believe that the activation of acid leukokinin by proteolytic release from the kininogen substrate is not a function of true cathepsin D, (i.e. acid protease with a pH optima of 3.0 on denatured hemaglobin and an isoelectric point in excess of 5.5) but rather is a property confined to that acid protease which we have described with a similar pH optimum but with an isoelectric point of 4.2 (cathepsin H). We are currently attempting to establish unequivocally the role of cathepsin H and cathepsin D in the activation of the acid leukokinin system.

#### Summary

During the past year our efforts for the preceding five years have been borne out successfully in that we have been able to (1) isolate, purify and chemically characterize MIF (2) isolate, purify and characterize chemically lymphotactin and (3) isolate, purify and partially characterize a lysosomal permeability factor hitherto known as Skin Reactive Factor, and presumed to be lymphokine. (4) We have made the fundamental discovery that the acid leukokinin system is most probably not a cathepsin D activated kinin, but rather is the result of the activity of the hitherto unrecognized cathepsin, which we choose to call cathepsin H (i.e. isoelectric point of pH 4.2). The reader's attention should be drawn to the fact that the only purified lymphokines currently known are MIF and our newly-discovered lymphotactin, both of which were obtained courtesy of contracts from the Office of Naval Research.

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